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## Development of efficient and stable host-vector systems for molecular cloning in *Bacillus subtilis*

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## SUMMARY AND GENERAL DISCUSSION

The development of efficient molecular cloning systems in the Gram-positive micro-organism *Bacillus subtilis* based on plasmid vectors derived from *Staphylococcus aureus* has met with several difficulties. Frequently observed problems with these plasmids were structural and segregational instability, and the low efficiencies in random cloning of foreign DNA (shotgun cloning). The latter is generally manifested in low yields of clones and limited insert sizes. The main purposes of the investigations described in this thesis were to uncover the major factors interfering with efficient molecular cloning in *B. subtilis* and, with this knowledge, to develop efficient and stable host-vector systems for this organism.

In chapter I a general introduction is given dealing with several aspects concerning molecular cloning in *B. subtilis*.

Chapter II describes the construction of a *Bsu*M restriction-deficient ( $r^-$ ), highly transformable strain (6GM), and a new cloning vector (pHP13), which carries the replication functions of the cryptic *B. subtilis* plasmid pTA1060. Using this host-vector system, we analyzed the effects of *Bsu*M restriction and plasmid size on the efficiency of shotgun cloning of heterologous *Escherichia coli* DNA in competent cells.

The host-vector system 6GM-pHP13 showed efficiencies of shotgun cloning far superior to those obtained in previously described systems, using *S. aureus*-derived vectors. Clones were obtained at a high frequency ( $10^4$  per  $\mu$ g target DNA) and large inserts were relatively abundant (26% of the clones contained inserts in the range of 6 to 15 kb), which resulted in a high average insert length (3.6 kb). Furthermore, it was shown that *Bsu*M restriction affected the efficiency of shotgun cloning by reducing the transforming activity and structural stability of recombinant plasmids. The results also indicated that, with the host-vector system 6GM( $r^-$ )-pHP13, plasmid size was not an important factor in the efficiency of shotgun cloning.

In chapter III we have analyzed the efficiency of shotgun cloning in *B. subtilis* protoplasts, and compared the results with those previously obtained with competent cells. Shotgun cloning in *B. subtilis* protoplasts appeared to be far less efficient than that in competent cells. This was the case both for the yield of clones (10-fold less for protoplasts) and for the size of inserts obtained: large inserts were relatively rare in protoplasts (12%

of the clones contained inserts in the range of 6-9 kb) in comparison to competent cells (26% of the clones contained inserts in the range of 6-15 kb). One of the major factors contributing to the relatively low efficiencies of cloning in protoplasts appeared to be the negative correlation between plasmid size and transforming activity with ligated plasmid DNA.

In chapter IV we describe experiments aimed to extend the applicability of the pTA1060 series of vectors by developing a  $\beta$ -galactosidase  $\alpha$ -complementation cloning system for *B. subtilis*. This system was based on two unrelated compatible plasmids. The host contained the pWVO1-based plasmid pGHS1, which carries the constitutively expressed *lacZ* $\Delta$ M15 gene. The cloning vector was the pTA1060-based plasmid pHPS9, which carries a constitutively expressed translational fusion of the *B. pumilus* *cat-86* gene and the *lacZ* $\alpha$  gene from plasmid pUC9. This system offered several advantages over previously described cloning systems for *B. subtilis*: (1), the direct selection of recombinants; (2), the cloning of large heterologous DNA fragments with high efficiency; and (3), the availability of 6 unique target sites for direct clone selection (*Sph*I, *Nde*I, *Nhe*I, *Bam*HI, *Sma*I and *Eco*RI).

Chapter V describes major improvements of the  $\beta$ -galactosidase  $\alpha$ -complementation system cloning system. The *lacZ* $\Delta$ M15 gene was stably integrated in the *B. subtilis* chromosome and the efficiency of translation of this gene was improved. Furthermore, a new *lacZ* $\alpha$  complementing vector was constructed, containing 8 unique target sites for direct clone selection (pHPS10). Using the new host with the integrated *lacZ* $\Delta$ M15 gene (6GM15), it was shown that large heterologous DNA fragments (up to at least 29 kb) could be cloned with *lacZ* $\alpha$  complementing vectors containing the replication functions of the cryptic *B. subtilis* plasmid pTA1060, and that these inserts were stably maintained for at least 100 generations of cell growth. It was therefore concluded that pTA1060-based plasmids are far superior to the commonly used *S. aureus* vectors with respect to cloning efficiencies and structural stability.

At present we can only speculate about the reasons why in *B. subtilis* pTA1060-derived plasmids are superior to the commonly used *S. aureus* plasmids. Since both groups of plasmids replicate in a similar way (via a rolling-circle mechanism), it is unlikely that the observed differences are a direct consequence of their mode of replication (as proposed by other authors). A possible explanation for this is that the types of structural

instability shown to be stimulated by ss DNA, do not occur frequently enough to interfere seriously with molecular cloning of foreign DNA in pTA1060-based plasmids.

It has been shown that the *S. aureus* plasmids generate, in addition to ss DNA, aberrant replication intermediates of high-molecular-weight (HMW), if they carry foreign DNA inserts. The presence of pBR322 sequences (in shuttle vectors) greatly enhanced HMW DNA production (e.g. in pHV33, a pBR322-pC194 shuttle: more than 70% of the plasmid DNA appeared to consist in the HMW form). Preliminary results in our laboratory suggest that the pTA1060-based plasmids described in this thesis generate only small amounts of HMW DNA. Both ss and HMW DNA have been conceived to cause plasmid instability, possibly in an indirect way by interfering with cell growth. Physiological stress imposed by ss DNA might either be caused by induction of the SOB response, or, alternatively, essential host enzymes might be withdrawn from the cellular pool (such as single-strand binding protein [essential for chromosomal replication]; only 300 copies present per cell in *E. coli*). A significant difference between the two groups of plasmids is their copy number: 5 per chromosome equivalent for pTA1060; and from 20 to 50 for most of the *S. aureus* plasmids. Because of this difference, the putative physiological stress effects would be much stronger for the staphylococcal plasmids. Enlarging the plasmid size by molecular cloning would reinforce these effects (assuming that the copy number stays constant) and might even lead to inviable situations. A cell can "escape" from plasmid-imposed physiological stress by either loosing the plasmid (segregational instability), or rearranging the plasmid (structural instability).

Although for several problems with respect to molecular cloning in *B. subtilis* attractive solutions can now be offered with the systems described above, at least one limitation still remained. In plasmid-mediated transformation of *B. subtilis* competent cells, only plasmid multimers are active. This forms a serious limitation for efficient cloning, since the potential for generating such molecules during ligation of vector and target DNA is limited. As a consequence, the cloning of blunt-ended DNA fragments in competent cells is highly inefficient and cloning of DNA fragments having incompatible ends (forced cloning) is not possible.

Chapter VI describes experiments in which we adapted the host-vector system 6GM15-pHPS9 to support transformation with monomeric plasmid molecules. The advantages of the  $\beta$ -galactosidase  $\alpha$ -complementation system



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were combined with those of plasmid marker rescue transformation (PMRT), which involves the uptake of donor plasmid DNA by competent cells and the subsequent recombination with a (partially) homologous resident plasmid. This novel PMRT system was shown to offer considerable advantages over previously described PMRT systems: (1), the convenient direct selection of recombinants; (2), the ability to effectively transform *B. subtilis* competent cells with plasmid monomers, which enables the forced cloning of DNA fragments with high efficiency; (3), the availability of 6 unique target sites for direct clone selection (*Sph*I, *Nde*I, *Nhe*I, *Bam*HI, *Sma*I and *Eco*RI); and (4), the rapid segregational loss of the helper plasmid from the transformed cells.